Plant Resistance and Its Effect on the Peritrophic Membrane of Southwestern Corn Borer (Lepidoptera: Crambidae) Larvae

C. A. DAVES,[1] W. P. WILLIAMS,[2] F. M. DAVIS,[3] G. T. BAKER,[3] P.W.K. MA,[3] W. A. MONROE,[4] AND S. MOHAN[5]

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ABSTRACT The southwestern corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae), is a serious pest of corn, *Zea mays* L., in the southern United States. Corn germplasm lines with conventional genetic leaf-feeding resistance to this pest, the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), and other lepidopterans have been released to the public by USDA–ARS scientists located in Mississippi. Recent studies suggest the insect resistant lines disrupt the integrity of the peritrophic membrane of the fall armyworm. The objectives of this study were to investigate any morphological differences in the structure of the peritrophic membrane of southwestern corn borer larvae feeding on resistant and susceptible corn hybrids and to quantify the damage. Larvae were reared under field and laboratory conditions on three corn hybrids (two resistant and one susceptible). Scanning electron microscopy was used to examine the peritrophic membrane for abnormalities such as holes or tears and to count the holes or tears in the membrane. Differences in the degree of damage to peritrophic membrane of larvae fed on resistant and susceptible plants were not detected. Up to five distinct layers of the membrane were observed in each larva. Variation in the amounts of damage to the peritrophic membrane observed from larvae feeding on all plant material was high. Plant resistance adversely affects growth and development of southwestern corn borer larvae, and further investigations are needed to explain the role of plant resistance and its relation to peritrophic membrane in southwestern corn borer larvae.

KEY WORDS host plant resistance, peritrophic membrane, southwestern corn borer

The southwestern corn borer, *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae), has many documented host plants. However, corn, *Zea mays* L., is the most preferred host. Moreover, the southwestern corn borer is considered the fifth most important insect pest attacking corn in the United States (Knutson and Davis 1999). This pest can cause substantial yield losses (Davis and Williams 1994). Yield losses associated with southwestern corn borer are attributed to larval feeding on leaf, ear, and stalk tissues. Management practices for control and suppression of southwestern corn borer include cultural, chemical, biological control, and plant resistance.

Davis et al. (1973) reported the corn genotypes exhibiting leaf-feeding insect resistance to southwestern corn borer in exotic Antigua Grupo 2 germplasm. This exotic corn germplasm has been used by several researchers primarily to develop Mississippi (Mp) inbred lines and populations with leaf-feeding resistance (Scott and Davis 1981a, 1981b; Scott et al. 1982; Williams and Davis 1980, 1982, 1984, 2000, 2002; Williams et al. 1990a). This resistance has also been effective against other lepidopteran pests such as the fall armyworm, *Spodoptera frugiperda* (J.E. Smith); European corn borer, *Ostrinia nubilalis* Hübner; and the corn earworm, *Helicoverpa zea* (Boddie). The mechanisms of resistance in Mp inbred lines are nonpreference and antibiosis (Wiseman et al. 1981, 1983; Davis et al. 1989). The factors responsible for insect resistance may be biochemical, anatomical, or a combination. Hedin et al. (1984, 1990) and Callahan et al. (1992) reported biochemical differences in tissues of resistant and susceptible Mp inbred lines. Theses genotypes differed in the amounts crude fiber and residue, hemicellulose, crude protein, and some nonessential amino acids as in polypeptides located in the whorl tissue. Ng (1988)) and Davis et al. (1995) reported differences in anatomical characteristics in resistant and susceptible lines. Plant anatomical characters have been associated with insect resistance by

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[1] Department of Plant and Soil Sciences, Box 9555, Mississippi State University, Mississippi State, MS 39762.

[2] USDA–ARS Corn Host Plant Resistance Research Unit Box 9555, Mississippi State, MS 39762.

[3] Department of Entomology and Plant Pathology, Box 9775, Mississippi State University, Mississippi State, MS 39762.

[4] Electron Microscope Center, Box 9775, Mississippi State MS, 39762.

[5] Department of Biochemistry and Molecular Biology, Box 9659, Mississippi State, MS, 39762.

Norris and Kogan (1980), Southwood (1986), and Smith (1989).

Recent studies using fall armyworm and southwestern corn borer larvae suggest that the insect resistance in Mp inbred lines is caused by the accumulation of a unique 33-kDa cysteine proteinase in the whorl tissue (Pechan et al. 2000). Further investigation by Pechan et al. (2002) found that fall armyworm larvae not only suffered a reduction in weight when fed on resistant whorl tissue but also experienced severe damage to the peritrophic membrane (PM), which was described as holes, perforations, structural voids, or abrasions of various sizes. The most extensive damage was associated with the endoperitrophic layer, which is in direct contact with the food bolus. Pechan et al. (2002) concluded that PM damage is most likely the cause of the reduced larval weights. Other studies involving damage to the PM suggest that increased PM permeability disrupts nutrient and enzyme cycling between the endoperitrophic and ectoperitrophic spaces (Terra 2001, Bolognesi et al. 2001).

The PM consists of a network of chitin and proteins, with the other major proteins called peritrophins (Terra 2001). The midgut is divided into endoperitrophic (inside PM) and ectoperitrophic spaces (outside PM) (Terra 2001). The PM is thought to have several different functions. One of the most noted functions found in the literature is that of protection of the epithelial layer from mechanical damage as food passes through the gut. Peters (1992) suggested that one function is epithelial protection, but another more important function may be that it acts as a barrier to microorganisms. Other studies have also shared this concept (Tellam 1996, Lehane 1997). Terra (2001) described the specific functions of the PM in that it compartmentalizes the midgut lumen. Lehane (1997) describes the following functions of the PM: mechanical barrier, barrier to infection, acts as a filter, chemical protection, and compartmentalization of digestion in the midgut. Harper and Hopkins (1997) showed that secretion and formation of the PM in European corn borer occurs primarily in the anterior region of the midgut.

The findings of Pechan et al. (2002) with the fall armyworm suggest that the factor(s) involved in the leaf-feeding resistance of the Mp corn germplasm lines caused the PM of the fall armyworm larvae to be disrupted, thereby explaining the significant larval weight differences compared with those reared on susceptible corn lines. Because the fall armyworm-resistant Mp lines are also resistant to the southwestern corn borer, the current study was conducted to determine whether the PM of southwestern corn borer larvae were similarly affected. Because damage to the PM can be deleterious to the insect, the objective in this study was to investigate any morphological differences in the structure of the PM found in southwestern corn borer larvae reared on the Mp corn hybrids under field conditions and in laboratory bioassays. A second objective was to quantify any damage associated with the PM caused by the larvae feeding on resistant and susceptible corn leaf tissue.

Materials and Methods

Southwestern corn borer larvae were randomly collected from field and laboratory experiments in 2002 and 2003 to examine their PMs. The field experiments were conducted at the R. R. Foil Research Farm, Mississippi State, MS. Two resistant corn hybrids, Mp704 × Mp707 and Mp714 × Mp716, and one susceptible corn hybrid, Ab24E × SC229, were grown using standard corn production practices. Corn plants were artificially infested with southwestern corn borer neonate larvae, and larvae were collected at different sampling intervals. Southwestern corn borer neonates were obtained from the colony maintained by the USDA–ARS Corn Host Plant Resistance Research Unit. The newly hatched larvae were mixed with corn cob grit and deposited into the plant whorl by using a mechanical larval dispenser (Wiseman et al. 1980). The plants were infested when they reached the V8–V9 leaf stages as defined by Ritchie et al. (1986). The larvae collected from the field experiments were held in the refrigerator at 4°C for several hours in individual plastic cups until they could be weighed individually and then dissected and processed for the following microscopic procedures.

In addition, southwestern corn borer larvae also were collected from the laboratory experiments 14 d after infestation (DAI) and processed using the same microscopic procedures. Larvae from the laboratory experiments were reared on a diet containing lyophilized whorl tissue taken from plants in the V8–V9 growth stage and grown under field conditions. This bioassay procedure was the same as described by Williams et al. (1990b).

Transmission Electron Microscopy. Southwestern corn borer larvae used for transmission electron microscopy (TEM) were collected and dissected on the same day. The larvae that fed on diet containing lyophilized whorl tissue from each of the three corn hybrids were collected 14 d DAI from the 2002 laboratory bioassay. The digestive system (fore, mid, and hind) of the southwestern corn borer larvae was removed and fixed in 2.5% glutaraldehyde fixative, in 0.1 M phosphate buffer, pH 7.2, for 2 h at 4°C. After fixation, specimens were rinsed in the same buffer and postfixed in 2% OsO_4 in 0.1 M phosphate buffer for 2 h and then rinsed in distilled water and dehydrated in an ethanolic series. Specimens were infiltrated and embedded in Spurr's resin and polymerized at 70°C for 15 h (Hayat 1986). Semithin sections (0.5–1.0 μm) and thin sections (60–100 nm) were collected for both light microscopy and TEM, respectively. Light microscopy was used to determine tissue orientation and initial PM detection. Thin sections mounted on Formar-coated 200 mesh copper grids were double stained with uranyl acetate and lead citrate for transmission electron microscopy and examined and photographed in a JEOL 100 CX II TEM (JEOL USA, Peabody, MA) at 60 kV.

Light Microscopy. The southwestern corn borer larvae used for the light microscopy study were processed on the sampling day by removal of the head and

anus. Larvae were placed in fixative to allow penetration of all tissues within the entire gut. Larvae were fixed in formalin, acetic acid, alcohol and then dehydrated in a graded ethanol series before being embedded in paraffin. Citri-Solve (xylene substitute available from Fisher Scientific Co., Pittsburgh, PA) was used as a transition fluid between ethanol and paraffin. Serial sections (8 μm) were cut on a rotary microtome. The sections were adhered to slides using Haupt's gelatin adhesive (VWR, West Chester, PA) and then stained with hematoxylin and eosin (Hayat 1981). The thickness of the epithelial layer in the midgut was measured in three locations. Three cross sections of each midgut were measured at approximately the 2, 6, and 10 o'clock positions. The average thickness was generated using nine measurements from each larva.

Scanning Electron Microscopy (SEM). *2002 Field Larvae.* In total, 21 larvae were collected in the field and processed for observation by using the scanning electron microscope. All larvae were collected 21 DAI. Eleven of the larvae were collected from the susceptible corn hybrid Ab24E × SC229. There were 10 larvae in total collected from resistant corn hybrids. Three larvae were collected from Mp704 × Mp707, and seven larvae were collected from Mp714 × Mp716. In total, 92 SEM photographs were examined.

2003 Field Larvae. In total, 29 larvae were collected from the field and processed for observations by using SEM. All larvae were collected 21 DAI. Nine of the larvae were collected from the susceptible corn hybrid Ab24E × SC229. Ten larvae were collected from each of the resistant corn hybrids Mp704 × Mp707 and Mp714 × Mp716. In total, 80 SEM photographs were examined.

2003 Laboratory Bioassay. In total, 30 larvae were collected and processed for observations by using SEM, and all larvae were collected 14 DAI. Eight of the larvae were collected from diet containing lyophilized whorl tissue from the susceptible corn hybrid Ab24E × SC229. In total, 22 larvae collected from the two resistant corn hybrids. Twelve larvae were collected from diet containing lyophilized whorl tissue from Mp704 × Mp707, and 10 larvae were collected from diet containing lyophilized whorl tissue of Mp714 × Mp716. In total, 120 SEM photographs were examined.

The following procedure is similar to that used by Pechan et al. (2002). The gut from larvae used for SEM study were removed and gently teased open to expose the food bolus and PM in the midgut. The digestive system of each insect was removed and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and stored at 4°C. After fixation, specimens were rinsed in buffer, postfixed in 2% OsO_4 in 0.1 M phosphate buffer for 2 h, and then rinsed in distilled water, dehydrated in a graded ethanol series, and critical point dried in a Polaron E 3000 Critical Point Dryer (Quorum Technologies, Newhaven, United Kingdom) (Hayat 1981). Specimens were mounted on aluminum stubs with double-sided carbon tape; sputter coated with gold palladium in a Polaron E 5100 sputter coater (Quorum Technologies, Guelph, Ontario, Canada), and viewed in a Zeiss SMT Stereoscan 360 scanning electron microscope (Carl Zeiss, Thornwood, NY) at an accelerating voltage of 15 kV. Images were recorded on Polaroid Type 55 film (Polaroid, Cambridge, MA).

The numbers of holes or tears observed in the PM were counted using two methods. The initial count included all damaged areas (holes or tears) regardless of shape or size. In the second attempt to quantify the damaged areas of the PM, only holes or tears that measured $\approx$1 mm^2 and larger were recorded. All data were subjected to analysis of variance (SAS Institute 1999–2001), and means were separated using Fisher protected least significance difference (LSD) test (Steel and Torrie 1980).

Results

Transmission Electron Microscopy. Larvae were processed and prepared using standard TEM protocols. Preliminary results revealed no staining or very little staining of the PM. Further observations of the PM were not consistent among samples. In some samples, the PM did not take up the stain and was thus not observed. In other samples, only small sections of the PM were observed, and in many of these samples it manifested as a faint line in some areas surrounding the food bolus. Efforts to determine damage or disruptions to the PM by using this technique were inconclusive in 2002. Further examinations using this technique were discontinued.

Light Microscopy. *2002 and 2003.* Southwestern corn borer larvae collected from the field were sectioned and stained for viewing. In 2002, larvae were collected in the field at 10, 14, and 21 DAI. In 2003, larvae were collected from the field at 21 and 28 DAI. In total, 85 larvae were collected from the field during this study. All efforts to use these larvae for detection of disruptions to the PM of the southwestern corn borer were inconclusive. The PM was not observed in all larvae, and in some larvae, only sections of the PM were visible. Because of the lack of staining and the difficulties in observing the PM clearly, all efforts to use this technique for observing damage or disruptions to the PM were terminated. The midgut epithelial layer, food bolus, and plant material were observed in the midguts of these larvae. Because the epithelial cells are exposed to the food contents found within the gut, they are a possible site for plant defense proteins (Peumans and Van Damme 1995). They suggested that binding of a plant defense protein to a glycoprotein receptor may result in the insect being repelled, retarded in its growth, or even killed. In 2002, larvae collected 10 DAI feeding on the resistant hybrid Mp704 × Mp707 had significantly thinner midgut epithelial layer than larvae feeding on the other corn hybrids ($F = 1.8$; df = 3, 5; $P = 0.0091$) (Table 1). Statistical analysis of southwestern corn borer larvae at 14 and 21 DAI revealed no significant differences in thickness of the midgut epithelial layer (Table 1).

In 2003 the average thickness of the midgut epithelial layer of larvae collected from the three hybrids was

Table 1. Average thickness (mean ± SD) in micrometers of the midgut epithelial layer from southwestern corn borer larvae collected from corn plants grown in the field in 2002 and 2003

Hybrid	Plant resistance classification	DAI 2002						DAI 2003			
		n	10	*n*	14	*n*	21	*n*	21	*n*	28
Ab24E × SC229	Susceptible	15	88 ± 16a	5	94 ± 24a	4	99 ± 24a	7	87 ± 26a	2	107 ± 27a
Mp704 × Mp707	Resistant	5	75 ± 13b	7	97 ± 18a	4	84 ± 48a	4	90 ± 30a	7	84 ± 26b
Mp714 × Mp716	Resistant	5	88 ± 21a	4	103 ± 19a	5	106 ± 22a	3	93 ± 32a	8	106 ± 27a

Means in columns followed by the same letter do not differ at $P > 0.05$ according to Fisher LSD test. Measurements were taken using light microscopy.

not significantly different at 21 DAI. However, southwestern corn borer larvae reared on the resistant hybrid Mp704 × Mp707 had a significantly thinner midgut epithelial layer than larvae feeding on the other two corn hybrids ($F = 5.11$; df = 2, 4; $P = 0.0285$) (Table 1).

Scanning Electron Microscopy. *2002 Field Larvae.* Some of the larvae observed in this study had fully filled PMs, others were only partially filled, and some did not contain a food bolus. Figure 1A shows the midgut of a larva reared under field conditions in which the PM was filled with a food bolus. Figure 1B represents a midgut that is partially filled with a food bolus. Distinct layers of the PM also could be observed as shown in Fig. 2. In one larva, five distinct layers of PM were observed (Fig. 2B). Plant material, such as trichomes, vascular bundles, and leaf material were observed in the food bolus of some southwestern corn borer larvae (Figs. 3 and 5). Damage or disruptions to the PM observed in this experiment varied among larvae reared on susceptible and resistant corn hybrids. The damage observed can be described as holes or tears in the PM, and in some photographs the plant material is observed protruding through the PM (Fig. 4). The number of holes or tears in the PM was counted and comparisons were made among larvae

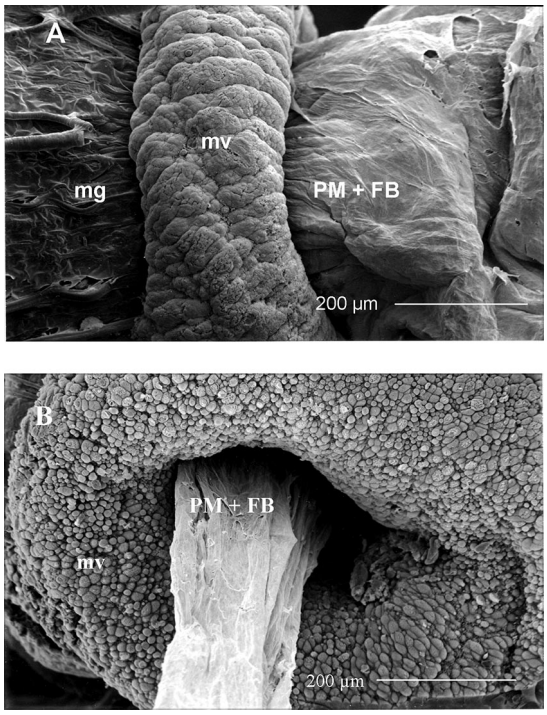


Fig. 1. Scanning electron micrographs showing southwestern corn borer midgut and PM. Larvae were reared on corn plants in the field in 2002. (A) Excised midgut (mg) from southwestern corn borer larvae reared on the susceptible corn hybrid Ab24E × SC229 showing the exposed microvilli (mv), and PM surrounding the food bolus (FB) (magnification 110×). (B) Excised midgut from a southwestern corn borer larvae reared on the resistant corn hybrid Mp714 × Mp716 showing microvilli (mv), and PM surrounding food bolus (magnification 152×).

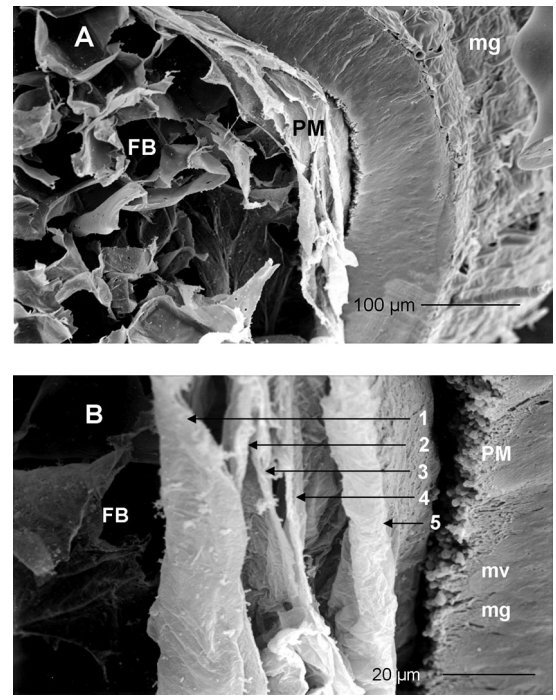
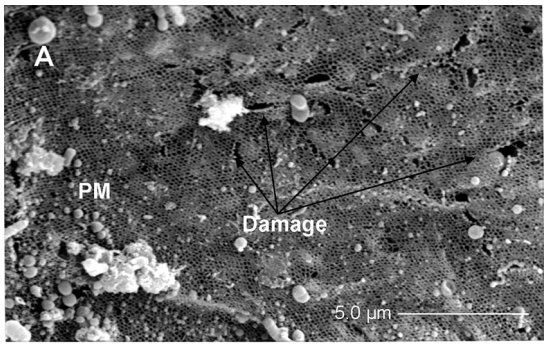


Fig. 2. SEMs showing southwestern corn borer midgut and layers of PM. Larvae were collected from field experiments conducted in 2002. (A) Excised midgut (mg) from a southwestern corn borer larvae reared on the resistant hybrid Mp704 × Mp707 showing the PM surrounding the food bolus (FB) (magnification 221×). (B) Excised midgut from a southwestern corn borer larvae reared on the resistant hybrid Mp704 × Mp707 showing microvilli (mv) and five distinct layers of PM surrounding the food bolus (FB) (magnification = 1,160×).



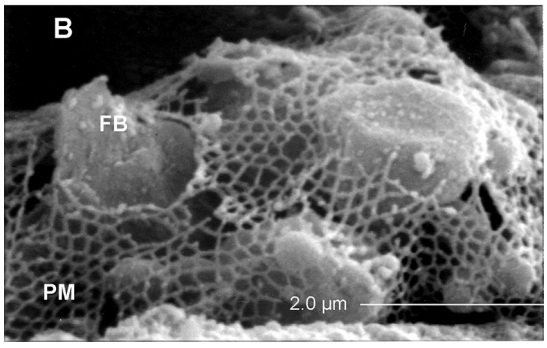
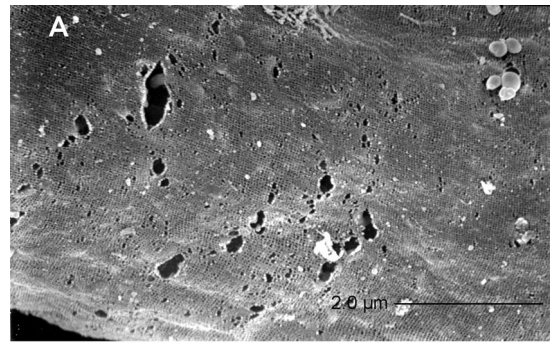


Fig. 3. SEMs showing southwestern corn borer PM and damaged areas. Larvae were collected from field experiments conducted in 2002. (A) PM from southwestern corn borer larvae reared on the susceptible hybrid Ab24E × SC229 showing the PM surrounding the food bolus with damage (magnification 5,760×). (B) PM from southwestern corn borer larvae reared on the susceptible hybrid Ab24E × SC229 showing a network of PM with the food bolus (FB) extruding through the PM (magnification 20,900×).



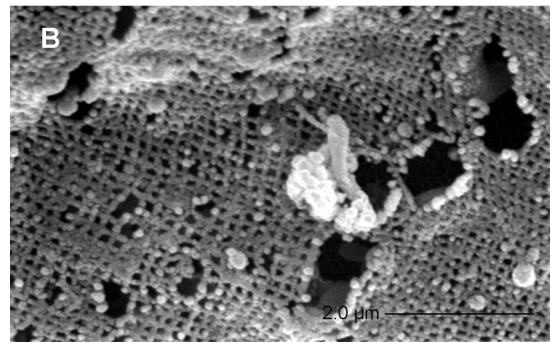


Fig. 4. SEMs showing southwestern corn borer PM and damaged areas. Larvae were collected from field experiments conducted in 2002. (A) PM from a larvae reared on the resistant hybrid Mp704 × Mp707 showing the PM with damaged areas (magnification 3,360×). (B) PM from a larvae reared on the resistant hybrid Mp704 × Mp707 PM with damaged areas (magnification 12,900×).

feeding on the different corn hybrids. When all holes or tears in the PM were counted the larvae from the susceptible corn hybrid averaged 3.4 damaged areas per cm^2. Larvae feeding on the two resistant hybrids Mp704 × Mp707 and Mp714 × Mp716 averaged 3.0 and 3.6 damaged areas per cm^2. There were no statistical differences observed among the larvae from the corn hybrids for damaged PM areas. A count of the damaged areas ≈1 mm^2 and larger also was attempted to quantify the amount of damage observed to the PM. No significant differences among the larvae reared on the three hybrids were observed.

In 2003, observations of the PM and damaged areas of the PM were similar to those in 2002. Some larvae had a larger food bolus than others, whereas other larvae did not have a food bolus present. The amount of damage to the PM was slightly higher in 2003 but not statistically different. Larvae fed on the susceptible hybrid averaged 3.3 damaged areas per cm^2, whereas larvae feeding on the two resistant corn hybrids averaged 4.8 (Mp704 × Mp707) and 6.4 (Mp714 × Mp716) damaged areas per cm^2. There were no significant differences in damaged areas of PM among larvae feeding on the three corn hybrids. Damaged areas ≈1 mm^2 and larger also were counted, and no significant differences were observed.

2003 Laboratory Bioassay. Southwestern corn borer larval midguts were examined for damaged areas or disruptions to the PM surrounding the food bolus. In this experiment all of the larvae had a food bolus present. Again, there were no significant differences among the PMs from larvae feeding on the different corn hybrids. Southwestern corn borer larvae feeding on the susceptible hybrid averaged 0.8 damaged areas per cm^2, and larvae feeding on the two resistant hybrids averaged 2.6 (Mp704 × Mp707) and 1.1 (Mp714 × Mp716) damaged areas.

Discussion

In this study, attempts to examine the PM were made using TEM, light microscopy, and SEM. However, the TEM and light microscopy techniques used in this study were not adequate when examining the damaged PM areas or disruptions that may have been present. This was because of a lack of staining of the PM. Therefore, detection of the PM using these techniques was very poor, and these samples were omitted. Using SEM techniques, damaged areas and or disruptions to the PM were clearly visible. Observations of the midgut, PM, and the food bolus were possible.

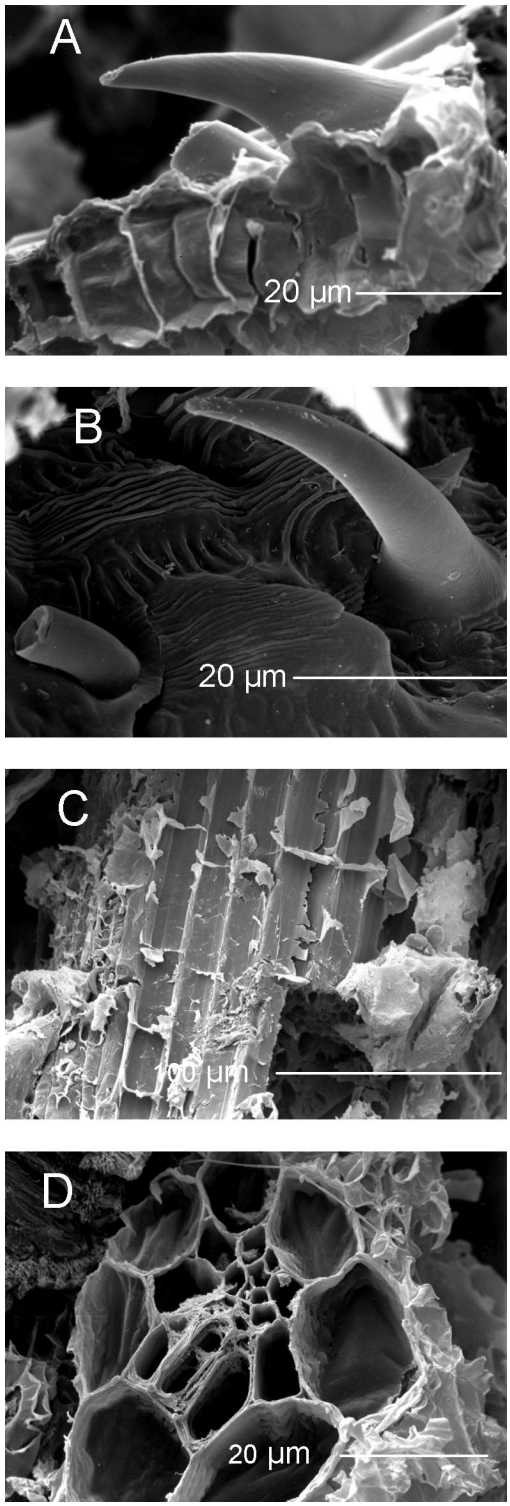


Fig. 5. SEMs showing plant material found in the food bolus of southwestern corn borer larvae reared on diet containing lyophilized whorl tissues of leaf feeding resistant and susceptible corn hybrids. (A) Plant material (trichome) found in food bolus of a southwestern corn borer larvae feeding on the resistant hybrid Mp704 × Mp707 (magnification 953×). (B) Plant material (trichome) found in food bolus of a southwestern corn borer larvae feeding on the susceptible hybrid Ab24E × SC229 (magnification 1,410×). (C) Plant material found in the food bolus of a southwestern corn borer larvae feeding on the susceptible hybrid Ab24E × SC229 (magnification 381×). (D) Plant material (vascular bundle) found in the food bolus of a southwestern corn borer larvae feeding on the resistant hybrid Mp704 × Mp707 (magnification 1,200×).

However, a food bolus was not present in all tested larvae. Two possible explanations for the differences observed regarding the presence of a food bolus may be due to the short delay in processing of the insects after recovery from the plant or because some larvae had just completed a molt. During the molting process, there is a pause in feeding. Efforts to quantify the number of damaged areas in the PM failed to reveal significant differences among larvae feeding on susceptible versus resistant plant material. Variation in the amounts of damage to PM observed from larvae feeding on all plant material was high. Larvae feeding on both resistant and susceptible hybrids sustained varying degrees of damage as illustrated in Figs. 1–4. Extreme damage to the PM was found when larvae were reared on both susceptible and resistant corn hybrids. There was no indication by observation of the PM or by the amount of damage to the PM that could be used to determine whether the larvae had been feeding on resistant or susceptible plant material. Southwestern corn borer larvae were examined at high and low magnifications, and the damage observed was not consistent among larvae feeding on any of the corn hybrids. The damaged areas were not restricted to any one layer of the PM. Observations revealed damaged areas on all layers of the PM examined in this study. However, not all larvae examined had the same number of layers of PM. In this study, five distinct layers of PM were observed in only one larva.

In the current study, the PM of southwestern corn borer larvae were examined to determine whether larvae feeding on the susceptible and resistant corn hybrids exhibited differences in the amounts of physical damage to the PM. Pechan et al. (2002) suggested that differences in weight gain among fall armyworm larvae feeding on Mp resistant versus susceptible plant material may be due to disruptions in the PM or by improper formation of the PM. Plants from the Mp resistant inbreds or single crosses (using the Mp inbreds as parents) have been shown to contain higher levels of a 33-kDa cysteine protease than susceptible inbreds or single crosses (Pechan et al. 2000). Pechan et al. (2000) reported that Mp resistant plants accumulate this 33-kDa cysteine protease at the feeding site within 1 h of fall armyworm feeding, and they found it was most abundant in the yellow-green tissue of the mid-whorl, which is the preferred feeding site of the fall armyworm and southwestern corn borer larvae.

Plant resistance adversely affected the growth and development of southwestern corn borer larvae.

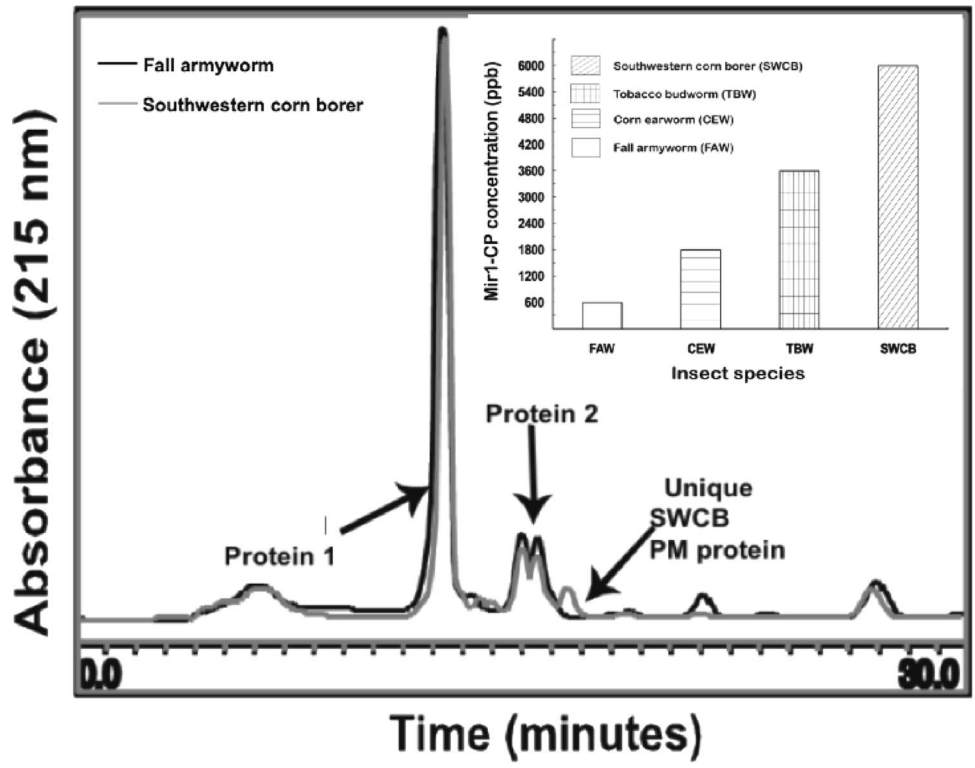


Fig. 6. Comparative analysis of PM proteins from southwestern corn borer and fall armyworm larvae by using size exclusion high-performance liquid chromatography.

Pechan et al. (2002) suggested that reduction in weight of FAW larvae feeding on resistant plant material was probably due to physical disruption of the PM, impaired development of the PM caused by the presence of the 33-kDa cysteine protease, or both.

A significant difference in growth and development of southwestern corn borer reared on resistant single crosses was observed in the field and laboratory studies (Daves et al. 2007). The degree of plant damage caused by southwestern corn borer was also significantly less on the resistant corn hybrids (Daves et al. 2007). However, observations of the PM from larvae feeding on resistant versus susceptible plant material failed to reveal significant differences. Differences in the PM protein composition of southwestern corn borer and fall armyworm have been shown (Fig. 6). Our data suggest that the southwestern corn borer has an additional PM protein not found in the fall armyworm. In an independent study, Mohan found that the southwestern corn borer requires a 10-fold higher dose of Mir1-CP (cysteine protease) compared with the fall armyworm to obtain an equivalent reduction in relative growth rate (Mohan 2006). Mohan et al. (2006) reported the PM of fall armyworm was the most effected of all species tested when treated with Mir1-CP. The PM of European corn borer was not affected by the Mir1-CP treatments. Further investigations of the role of plant resistance as related to the PM are needed to help explain the possible effects that plant resistance has on reducing southwestern corn borer growth and development.

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